AD_____

Award Number: W81XWH-11-1-0324

TITLE: Molecularly Targeted Dose-Enhancement Radiotherapy Using Gold and Luminescent Nanoparticles in an Orthotopic Human Prostate Cancer Rat Model

PRINCIPAL INVESTIGATOR: Kwang Song, Ph.D.

CONTRACTING ORGANIZATION: University of Texas Southwestern Medical Center
Dallas, TX 75390-9187

REPORT DATE: October 2013

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2013	Annual Summary	25 July 2011 – 24 July 2013

4. TITLE AND SUBTITLE

Molecularly Targeted Dose-Enhancement Radiotherapy Using Gold and Luminescent Nanoparticles in an Orthotopic Human Prostate Cancer Rat Model

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-11-1-0324

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Kwang Song, Ph.D.

E-Mail: ksong1@hfhs.org

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Texas Southwestern Medical Center
Dallas, TX 75390-9187

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Dose enhancement effect from gold nanoparticles with kV energy x-ray radiation had been evaluated on several cell lines and two different sizes of nanoparticles. In the past, 50 nm gold nanoparticles and several cell lines, such as DU-145, PC-3, and HeLa had been used for this study. All experimental procedures were followed the published protocols and modified as necessary. From these combinations, the dose enhancement effect was very minimal and even considered as negligible. Nanoparticle size has been switched to 100 nm which is also spherical shape and made of the same material as 50 nm nanoparticles. The dose enhancement of 100 nm nanoparticles have been evaluated on MDA-MB-231 breast cancer cell line with clonogenic assay. We observed that free nanoparticles around cells in tissue culture medium, not within the cells, have contributed dose enhancement at great amount. This dose enhancement was not observed in the 50 nm nanoparticles. Therefore, dose enhancement effect from gold nanoparticles is dependent on both the particle size and cancer cell lines.

15. SUBJECT TERMS
Radiation therapy, Dose enhancement, Gold nanoparticles, Orthotopic prostate tumor, Rat model

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	17	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Introduction

Nanoparticle researches have been active fields last decades because of their physical and functional characteristics, such as their size, capability of surface modification, long circulation time in a body, and various constituent materials. In particular, nanoparticles have more attentions since they are biocompatible and can be used for both cancer detection and treatment. For these purposes, nanoparticles are engineered in their shape and surface condition to be optimized for specific goals including maximizing the circulation time and the accumulation. Other active studies are functionalization of nanoparticles. Through these processes, nanoparticles have different functions, such as targeted drug delivering, sensing, and treating compared to the bare nanoparticles. In this report, we have studied the dose enhancement effect from gold nanoparticles with x-ray irradiation in the cancer treatment. This is also called dose enhanced radiation therapy. The dose enhancement effect is attributed to photoelectric effect in which electrons are released from gold atoms by absorbing energy from incoming x-ray photons. The photoelectric effect is proportional to the atomic number of absorbing materials and inversely proportional to energy of incoming photons. Therefore, higher atomic number materials (gold, lead, and etc.) will release more electrons than low atomic number materials (tissues, water, and etc.). Gold is the ideal material for the biomedical applications specially for the dose enhancement because of its biocompatibility and higher atomic number. The dose enhancement effect from gold nanoparticles has been studied in prostate cancer cell lines, such as DU-145, PC-3, and LNCap. The size of gold nanoparticles was in the range of 50 nm or 100 nm with/without PEG coating. To verify the integrity of gold nanoparticles fabricated in our collaborator and experimental protocols used in our laboratory, other cell lines, such as cervix cancer cell line (HeLa) and breast cancer cell line (MDA-MB-231), were also employed. The experiments with other cell lines provided good information to do problem-solving occurred in the experiments on prostate cancer cell lines. In this report, difficulties observed in experiments, procedures to resolve them, new discoveries, and possible solutions will be discussed.

Specific Aims

Three specific aims were proposed:

Aim 1: To analyze the dose enhancement effect of gold nanoparticles *in vitro*.

Aim 2: To develop targeted dose enhancement radiotherapy in an orthotopic human prostate tumor rat model using gold nanoparticles.

Aim 3: To evaluate the feasibility of x-ray excited luminescent nanoparticles as a contrast agent and radiosensitizer.

We have conducted experiments and have made progress toward specific Aim 1. Details for experimental results are described in the following sections.

Following tasks were proposed for the second year.

Task 1 (Specific Aim 2.2): Months 8-15
100 µL of gold nanoparticles, PEGylated and mAbs-conjugated, will be administered intravenously into the tail vein of two group 6 nude rats for accumulation experiments. The kinetics of gold nanoparticles will be determined using bioluminescence imaging. Subsequently, the inductively coupled plasma mass spectrometry (ICP-MS) method will be used for quantitative measurement of total accumulation. All work will be performed by the PI, with the exception of ICP-MS which will be performed by Dr Chen's lab.

Task 2 (Specific Aim 2.3): Month 8-15
Three groups of 9 nude rats bearing orthotopic human prostate cancer will be assigned for the evaluation of the x-ray phototherapy treatment method. One group is a control which will not have radiation. The other two groups will have the radiation therapy with and without the mAbs-conjugated gold nanoparticles. Treatment outcome will be monitored periodically using bioluminescence imaging following luciferin administration. The PI will do radiation delivery and assessment of treatment outcome under the supervision of Dr. Saha.

Task 3 (Specific Aim 3): Month 17-22
Evaluate the feasibility of x-ray excited luminescent nanoparticles as a contrast agent and radiosensitizer.

Key research accomplishments

Dose enhancement effect from gold nanoparticles has been investigated further with different size of nanoparticles and different cancer cell lines. Dose enhancement effect can be evaluated in various ways. Here, we have employed immunofluorescence imaging, MTT assay, and clonogenic assay, which are typical assays used in radiation biology and pharmaceutical research fields. In the project, the first hypothesis was that the dose enhancement effect is general and universal so that it can be applied to prostate cancer cell lines. This hypothesis was attainable because dose enhancement from gold nanoparticles is attributed to photoelectrons, which are not considered different from secondary electrons generated by photons in LINAC based radiation therapy. Therefore, dose enhanced therapy from this technique is another type of a dose escalated radiation therapy. Literatures also describe the dose enhancement from gold nanoparticles. However, our experiments with experimental protocols, nanoparticles, and cell lines presented the results much different from our initial hypothesis. Therefore, our study was focused more on identifying what protocols, what types of nanoparticles, and which cancer cell lines are to have more dose enhancement effect from gold nanoparticles. In this section, we will present our results from the experiments, problems, trouble-shooting, and potential solutions.

1. Verification of cellular uptake of gold nanoparticles using an optical microscopy

General understanding in literatures about this technique is that internalized gold nanoparticles play a major role to generate more photoelectrons from gold nanoparticles and to make more DNA double strand breaks (DSBs). Subsequently, more double strand breaks will be ended up with more cell death. The understanding of more cell death is based on Auger electrons which have shorter traveling ranges. In order for Auger electrons to make DNA DSBs, they should be located close to cell nuclei. Therefore, we tried qualitative evaluation of the cellular uptake of gold nanoparticles on DU-145, PC-3, LNCap, HeLa, and MDA-MB-231 using immunofluorescence imaging. HeLa and MDA-MB-231 cell lines were employed for the purpose of identification of the problems on prostate cancer cell lines occurred in clonogenic assays.

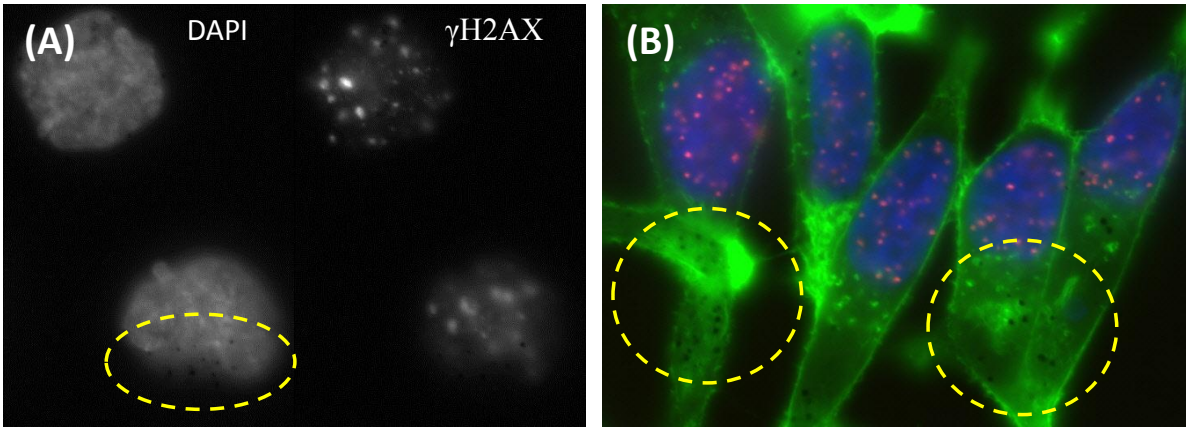


Figure 1. Qualitative evaluation of cellular uptake of 50nm nanoparticles using immunofluorescence staining. (A) DAPI and γH2AX staining for cell nuclei and DSBs in DU-145, respectively. (B) Phalloidin staining for actin protein in cell cytoplasm in LNCap prostate cancer cells in addition to DAPI and γH2AX staining. The dotted circles indicate nanoparticles visualized with black dots. Images were obtained using immunofluorescence microscopy.

We first tried 50 nm spherical gold nanoparticles for dose enhancement study because this size of nanoparticles has been studied to be an optimal size for cellular uptakes. Immunofluorescence staining (DAPI) has been used to qualitatively evaluate the cellular uptake of nanoparticles. As shown in figure 1, gold nanoparticles are distributed in cell cytoplasm as clusters, in which many nanoparticles were accumulated together and could be visualized in immunofluorescence microscopy. Since gold nanoparticles highly absorb optical photon energy without emission and scatter, they are shown as dark spots. Gold nanoparticles in 50 nm size were not imaged in regular optical microscopy while being imaged in immunofluorescence microscopy (an image is not presented). However, with 100 nm gold nanoparticles, an optical microscopy could resolve the clustered nanoparticles (no single nanoparticle) in the cell cytoplasm. In figure 2, the accumulation of gold nanoparticles is shown in both 10X magnification and 40X magnifications. In the 40X magnification, the accumulated nanoparticles are shown in the cell cytoplasm as dark spots. An optical microscopy cannot resolve an individual nanoparticle because of its limited resolving power. This uptake evaluation of 100 nm gold nanoparticles on MDA-MB-231 cancer cell line was performed to identify the problems on application of the dose enhanced technique to prostate cancer cell lines.

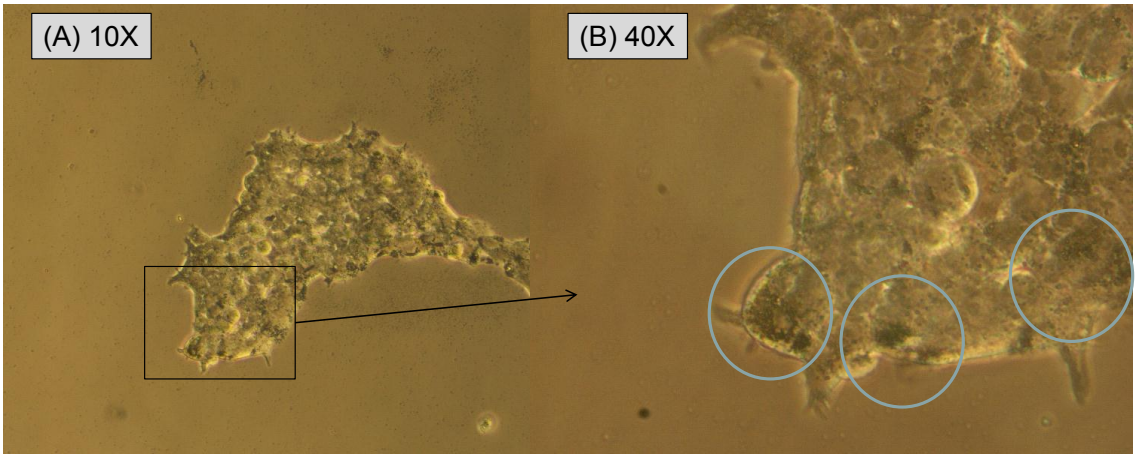


Figure 2. Optical microscopy of MDA-MB-231 breast cancer cells incubated with 100 nm gold nanoparticles for 24 hours. (A) 10X magnification. (B) 40X magnification. The circles indicate highly accumulated regions of gold nanoparticles.

2. Immunofluorescence imaging and MTT assay for evaluation of dose enhancement

The accumulation of gold nanoparticles was qualitatively evaluated using an immunofluorescent microscopy for 50 nm nanoparticles and an optical microscopy for 100 nm nanoparticles. Gold nanoparticles were accumulated in the cell cytoplasm near nucleus. The distances of the accumulated sites of gold nanoparticles from the nucleus seem to be various upon cell type. It is reasonable to claim that because different cell types should have different mechanism of endocytosis and delivering mechanisms. On DU-145, PC-3, and LNCap prostate cancer cell lines, we performed γ-H2AX immunofluorescence assay and MTT assay, both of which are relatively easy and quick. HeLa cell line was also used for verification of the protocols because HeLa cells have been successfully used in multiple literatures for the dose enhancement study. While the procedures of these two assays are easy and quick for the evaluation of effectiveness of drugs and treatments, the assessment of the results is somewhat subjective. Therefore, an objective method such as clonogenic assay should be performed to confirm the effectiveness of the substance of interest in addition to immunofluorescence and MTT assays. Figure 3 is the immunofluorescence assay results showing the number of γ-H2AX foci with respect to dose (figure 3(A)) and time (figure 3(B)). From our immunofluorescence assay on DU-145 and LNCap, the dose enhancement effect was statistically insignificant between the control group (without gold nanoparticles) and the experimental group (with gold nanoparticles). We had performed other types of γ-H2AX assay with different energy and doses because photoelectric effect is inversely proportional to the energy of x-ray and the creation of foci is also various upon dose. Figure 3(A) shows the changes in number of γ-H2AX foci with 1Gy, 2Gy, and 4Gy. As expected, the higher dose created more foci than the lower dose. On the other hand, lower energy of 105 kVp but at the same dose as 225 kVp does not enhance the dose. Figure 3(B) is a typical γ-H2AX graph as a function of time with a single dose showing the repairing kinetics of DNA DSB, in which LNCap prostate cancer cell line was used. Again, the dose enhancement was not presented on the LNCap cancer cells, either. We had also performed many different setups for γ-H2AX assay, such as different incubation time of gold nanoparticles with cancer cells, different time point of cell fixation, and 3 dimensional foci counting. However, any of γ-H2AX immunofluorescence assays did not provide any support to claim the dose enhancement on DU-145 and LNCap prostate cancer cell lines.

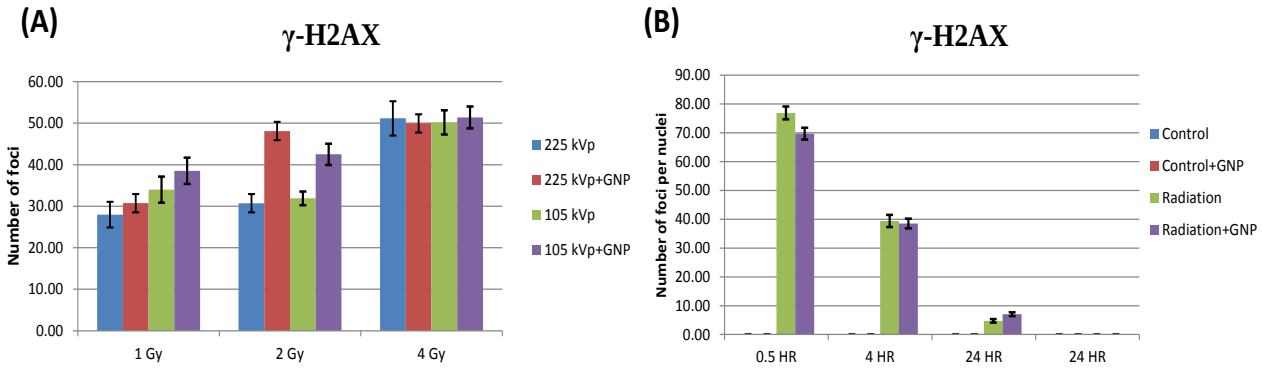


Figure 3. Immunofluorescence assay (γ-H2AX). (A) DU-145 prostate cancer cell line. Number of γ-H2AX foci as a function of radiation dose from 105 kVp and 225 kVp x-ray energy. (B) LNCap prostate cancer cell line. Number of γ-H2AX foci as a function of repairing time with 225 kVp x-ray radiation.

Since our observation from γ-H2AX assays did not support the claims by other groups in literatures, we had conducted MTT assays to reevaluate our protocols and techniques for *in vitro* experiments on DU-145, LNCap, and PC-3 prostate cancer cell lines. Figure 4 displays the cell viability of DU-145 and LNCap prostate cancer cells treated with gold nanoparticles and 225 kVp x-ray radiation. Before radiation, cells were incubated with 50 nm gold nanoparticles for 24 hours for sufficient intracellular uptake of nanoparticles. With the radiation dose of 0Gy, 4Gy, 8Gy, and 12Gy, the cell viability of both cell lines were decreasing as expected. However, there were no statistically significant differences between the control groups (without gold nanoparticles) and the experimental groups (with gold nanoparticles) in both cell lines. The same result was obtain from the PC-3 prostate cancer cell line (data is not presented). These experiments present that gold nanoparticles did not enhance the dose enough to make differences in cell viability.

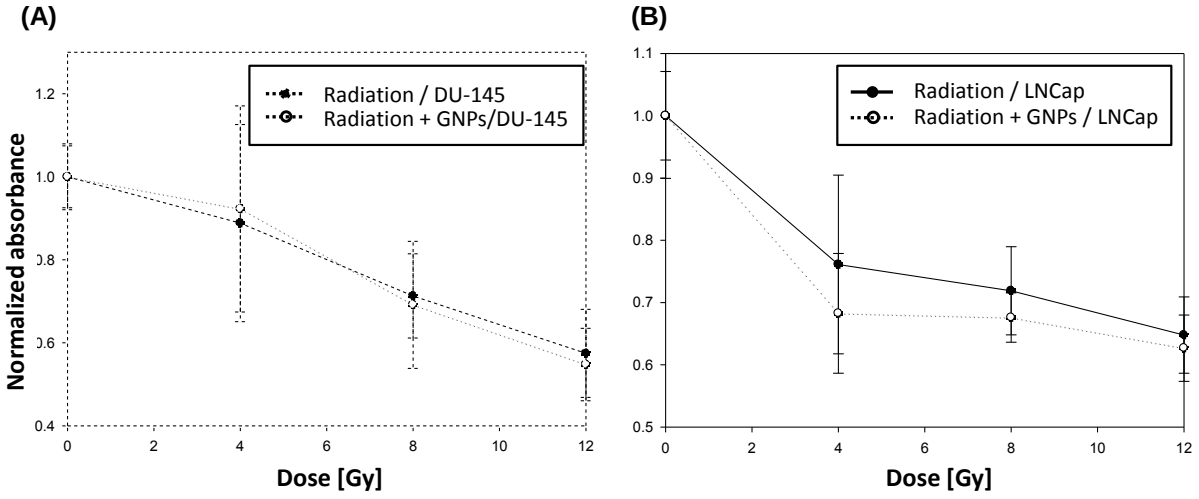


Figure 4. MTT assay for DU-145 (A) and LNCap (B). Cells were incubated with 50 nm gold nanoparticles for 24 hours.

3. Clonogenic assay for evaluation of dose enhancement effects

1.1. Dose enhancement in prostate cancer cell lines and problems

As aforementioned, clonogenic assay is superior to both γ-H2AX and MTT assays in the aspect of objectivity and long term monitoring of cell survival. The γ-H2AX assay is typically done within 24 hours and the MTT assay takes around 72 hours. The former counts the DNA DSBs and the latter measures the cell viability. Both of them do not provide any information regarding cell reproducibility and quiescence. On the other hand, clonogeneic assay is

more objective and can evaluate both cell reproducibility and quiescence in long period of time. We had conducted large number of clonogenic assays for DU-145, PC-3, and LNCap prostate cancer cell lines. In addition, HeLa and MDA-MB-231 cancer cell lines were also selected to perform troubleshooting for the outcomes presented in the prostate cancer line dose enhancement study.

In this report, only DU-145 clonogenic assay result is presented because the other two cell lines, PC-3 and LNCap, failed to form colonies in *in vitro* conditions. Briefly describing the clonogenic assay protocol, cells in the experimental groups were incubated with gold nanoparticles for 24 hours and were plated to individual circular cell culture dishes with a predetermined number of cells which are expected to form optimal number of colonies after around 10 days. Three hours after plating, second round of gold nanoparticles was added to experimental groups to mimic realistic condition at the time of irradiation. Immediately after or a few hours later of the second nanoparticle administration, cells were exposed to x-ray irradiation with typical doses of 0Gy, 2Gy, 4Gy, 6Gy, and 8Gy with the energy of 225 kVp. Depending on the sizes of colonies, the dishes were typically harvested around 9 days after x-ray irradiation. The dishes were post-processed following the protocol which has been used in our radiation biology group, and colonies were counted manually or using a colony counter.

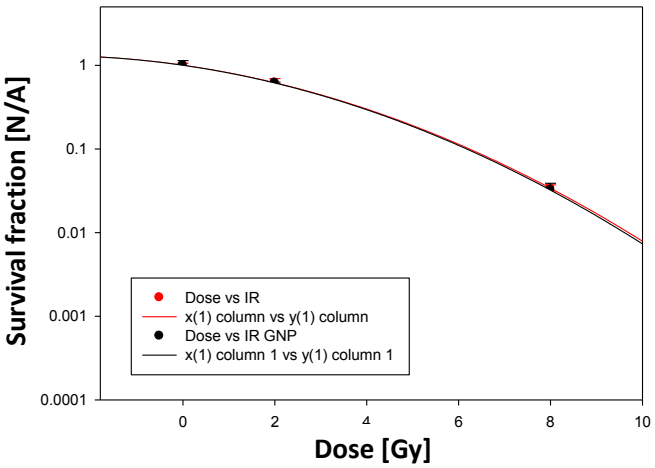


Figure 5. Survival fraction for DU-145 prostate cancer cell line obtained from a clonogenic assay. Dose vs IR indicates the control group without gold nanoparticles. Dose vs IR GNP means the experimental group with gold nanoparticles. The survival curve here was selected from one of large numbers of clonogenic assays for the example.

Survival fraction of DU-145 prostate cancer cells is presented in figure 5 with 0Gy, 2Gy, and 8Gy doses from 225 kVp x-ray irradiator. The red and black curves are the curve fittings of the survival fraction at each dose from the control and the experimental groups, respectively. In the figure 5, the dose enhancement is not observed at all. To further evaluate dose enhancement on DU-145, we also modified the parameters in the protocol, such as the incubation time with the first and second gold nanoparticles, concentration of nanoparticles, low temperature

during irradiation, different culture medium, and existence of second nanoparticles during irradiation. We had also tried two other protocols, so called immediate and delayed plating.

From all of clonogenic assays with different settings as mentioned, the dose enhancement effect on DU-145 cell line was not presented at all. HeLa cell line was also tested using clonogenic assay with variety of different settings aforementioned. However, the outcomes did not indicate dose enhancement. In this kind of experiments, there would be critical parameters which play major roles to generate more photoelectron resulting in more cell deaths. These parameters could be the size of nanoparticles, shape of nanoparticles, different mechanism of cell endocytosis, sensitivity of cells to small radiation dose increase, role of extracellular and intracellular gold nanoparticles.

1.2. Problem-solving for dose enhancement in prostate cancer cell lines

It was necessary for us to make a dramatic change on our experimental condition to resolve the difficulties that we had observed in our experiments on prostate cancer cell lines. By chance, we had performed clonogenic assays on MDA-MB-231 breast cancer cell line with 100 nm gold nanoparticles following the same protocol used for DU-145 prostate cancer cell line and others. The dose enhancement was statistically significant as shown in figure 6, where two different concentrations (1X and 2X) of gold nanoparticles were tried. As presented in other literatures and shown in figure 6, the higher concentration enhances cell killing effect than lower concentration. In addition, intracellular and extracellular gold nanoparticles were evaluated for their functions in dose enhancement. As a matter of convenience, the former is called 1^{st} GNPs because they were initially incubated with cells for 24 hours and the latter is indicated 2^{nd} GNPs because they were administered after 1^{st} GNPs right before x-ray radiation. Therefore, the 1^{st} GNPs were internalized and 2^{nd} GNPs were remained in the cell culture media at the time of irradiation. By this method, it can be evaluated that which gold nanoparticles inside or outside cells play a key role for the dose enhancement.

Another observation which is not indicated in literatures is that intracellular gold nanoparticles seem to have no contribution to dose enhancement at least in MDA-MB-231 cell line. In figure 6(A) and 6(B), the group containing both 1^{st} GNP and 2^{nd} GNP has less survival fraction than both radiation only group and the group containing 1^{st} GNP only. In addition, radiation only and 1^{st} GNP only groups do not show difference in survival fraction. It could be attributed to more gold nanoparticles in cell culture medium (extracellular nanoparticles) around cells than in cell cytoplasm (intracellular nanoparticles) or to synergistic effect from both intra- and extra-cellular nanoparticles. To make it clear, we had conducted another experiment in which an intracellular nanoparticle group (1^{st} GNP only) was compared with an extracellular nanoparticle group (2^{nd} GNP only). Figure 7 clearly indicates that intracellular gold nanoparticles do not enhance dose, but extracellular nanoparticles do. For these experiments, the concentration for the 1^{st} and 2^{nd} gold nanoparticles was the same. However, the amount of intracellular gold nanoparticles was expected to be less than the extracellular gold nanoparticles because of limited and equilibrating endocytosis. Whenever dose enhanced radiation therapy based on photoelectric effect is discussed, Auger electrons are the primary interest because they exert energy within a short distance in a burst format unlike photoelectrons lose energy gradually in long distances. However, in our experiments with 100 nm gold

nanoparticles on MDA-MB-231 cells, photoelectrons from extracellular gold nanoparticles turn out to be major players for the dose enhancement.

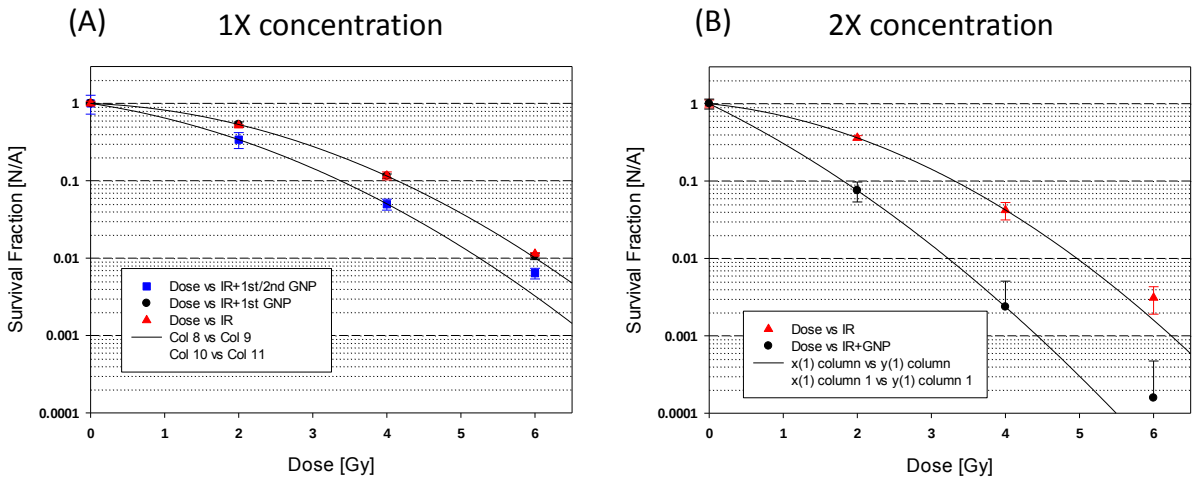


Figure 6. Survival fraction curves from clonogenic assays from the 100 nm gold nanoparticles applied on MDA-MB-231 with two different concentrations. 1X concentration: $2x10^8$ particles/ml. 1st GNP in (A): Intracellular nanoparticles. 2nd GNP in (A): extracellular nanoparticles. GNP in (B): intra- and extra-cellular nanoparticles.

We tried 100 nm gold nanoparticles on DU-145 prostate cancer cells as well. However, at the same concentration used for MDA-MB-231 cells, DU-145 could not form colonies and nearly a few cells were survived. This would be attributed to the larger size and greater amount of gold nanoparticles existed in extra- and intra-cellular spaces. This phenomenon also happened in the clonogenic assays when higher concentration of 50 nm gold nanoparticles was incubated with DU-145 cells. Due to the limited time left in the grant period, we could not find the optimal concentration for DU-145 specifically for 100 nm gold nanoparticles. It will be further studied to evaluate 100 nm size of gold nanoparticles for the dose enhancement on DU-145.

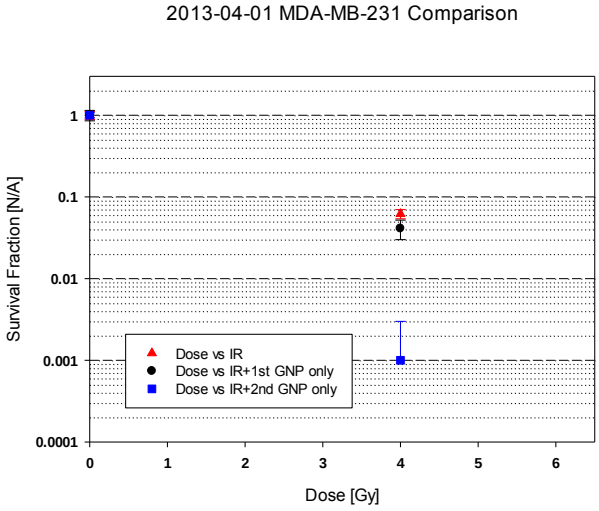


Figure 7. Survival fraction from clonogenic assay. Extracellular nanoparticles: 2nd GNP; Intracellular nanoparticles: 1st GNP.

1.3. Parameters that can affect dose enhancement

In literatures, the optimal size of gold nanoparticles for dose enhanced radiation therapy is claimed to be 50 nm because this size is the best for cellular uptake. We have also conducted experiments with 50 nm nanoparticles on DU-145, PC-3, LNCap, and HeLa. However, dose enhancement on those cell lines was not observed. Since we have observed dose enhancement from 100 nm gold nanoparticles on MDA-MB-231, we have tried 50 nm nanoparticles on MDA-MB-231 for clonogenic assay. The 50 nm nanoparticles did not show dose enhancement. From these experiment, we can conclude that gold nanoparticle based dose enhancement depends on the size of particles as well as cell lines. Table 1 summarizes the results of clonogenic assays with 50 nm and 100 nm gold nanoparticles on several cell lines. X stands for no dose enhancement, O represents dose enhancement, TBD indicates to be determined later. Fifty nanometer gold nanoparticles did not enhance dose on all of cell lines that we had evaluated. However, 100 nm gold nanoparticles show significant dose enhancement on MDA-MB-231 cell line. Even though dose enhancement was not observed in prostate cancer cell lines, the experience on MDA-MB-231 cell line greatly contributed for us to gain valuable information about nanoparticle applications and will provide clues to do problem-solving for the nanoparticle applications for prostate cancer. We will study further dose enhancement on prostate cancer using 100 nm gold nanoparticles.

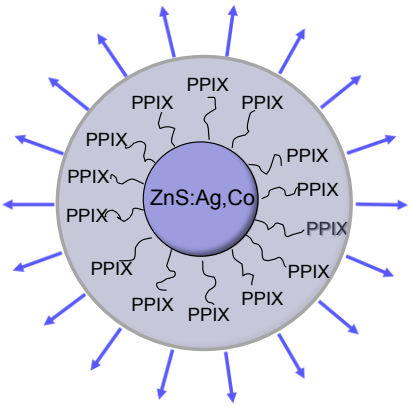
Protocol	size	DU-145	PC-3	HeLa	MDA-MB-231
Protocol 1	50nm	X	X	X	X
	100nm	TBD	TBD	X	O
Protocol 2	50nm	X	X	X	X
	100nm	TBD	TBD	X	O
Protocol 3/ Protocol 4	50nm	X	X	X	TBD

Table 1. Dose enhancement effect from 50 nm and 100 nm gold nanoparticles on different cancer cell lines.

4. Afterglow nanoparticles for photodynamic cancer therapy

Photodynamic therapy has been studied for cancer treatment in various lesions. However, its application is limited to shallow lesions where visible light can be delivered because of the limited penetration depth of light source. In photodynamic therapy, with the visible light excitation, photosensitizer creates free radicals which cause cell death. Our collaborator has been studying afterglow nanoparticles as an alternative light source to resolve the problem of limited light penetration. The nanoparticles have been developed to emit visible light by excitation of x-ray photon. Unlike visible light, x-ray phton has higher energy resulting in longer penetration power. The nanoparticles have

capability of glowing for a while even after the excitation is terminated, which is called afterglow. The nanoparticles are composed of ZnS:Ag,Co, Protophopyrin (PPIX), and PLGA. The core material, ZnS:Ag,Co, has afterglow effect. By using this nanoparticles, visible light can be delivered to the deeply seated tumor. The nanoparticles are also coated with PPIX, which is photosensitizer used in photodynamic therapy and generates free radical for cancer treatment. The nanoparticles are fabricated as shown in figure 7. Once the core component gets x-ray irradiation and it absorbs x-ray energy and emits light photon. The nanoparticles can still emit light even the excitation of x-ray stops. Therefore, it can excite the PPIX for longer time.



PLGA coated ZnS:Ag,Co+PPIX

Figure 8. Configuration of afterglow nanoparticles. Core is made of ZnS:Ag,Co and it has Protoporphyrin IX (PPIX) on its surface, and is coated with PLGA.

The emission spectrum of the after-glow nanoparticles by 360 nm UV lamp excitation is shown in figure 8A which ranges from around 400 nm and 700 nm wavelengths. Afterglow has a peak at 475 nm wavelength and its intensity is weaker than photoluminescence. Afterglow nanoparticles are also capable of emitting visible photon with x-ray excitation. Again, this is great feature to deliver x-ray energy to the deeply located afterglow nanoparticles which will finally emit light photon and excite PPIX. Figure 8B shows the intensity of emission of afterglow nanoparticles after 120 kVp and 250 kVp x-ray irradition. All images were obtained using a cooled CCD camera with 30 second exposure after 1, 2, 3, and 4 min x-ray excitation. With more x-ray excitation, higher intensity of light emission was observed.

The capability of afterglow nanoparticles for cancer treatment was evaluated using MTT assay. The afterglow nanoparticles were tagged with PPIX as described in figure 7 to enable photodynamic therapy. Five Gy of 120 kVp x-ray was delivered to different groups; control, nanoparticles only, PPIX only, and nanoparticles with PPIX coated. The cell viability from this experiment is shown in figure 9A. Afterglow nanoparticles with PPIX show the least cell viability. Figure 9B is fluorescence image of cells with afterglow nanoparticle incubation to assess

cellular uptake of nanoparticles. In figure 9(B)c, red color represents afterglow nanoparticles internalized to cell cytoplasm.

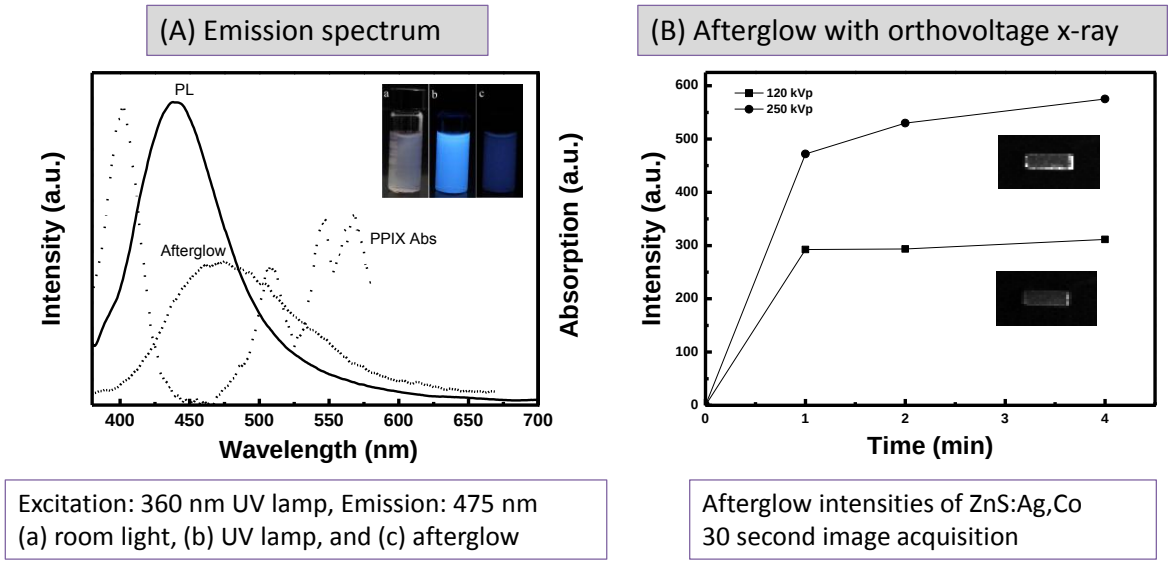


Figure 9. Emission characteristics of afterglow nanoparticles. (A) Emission spectrum by UV excitation. (B) light intensity of afterglow by x-ray excitation.

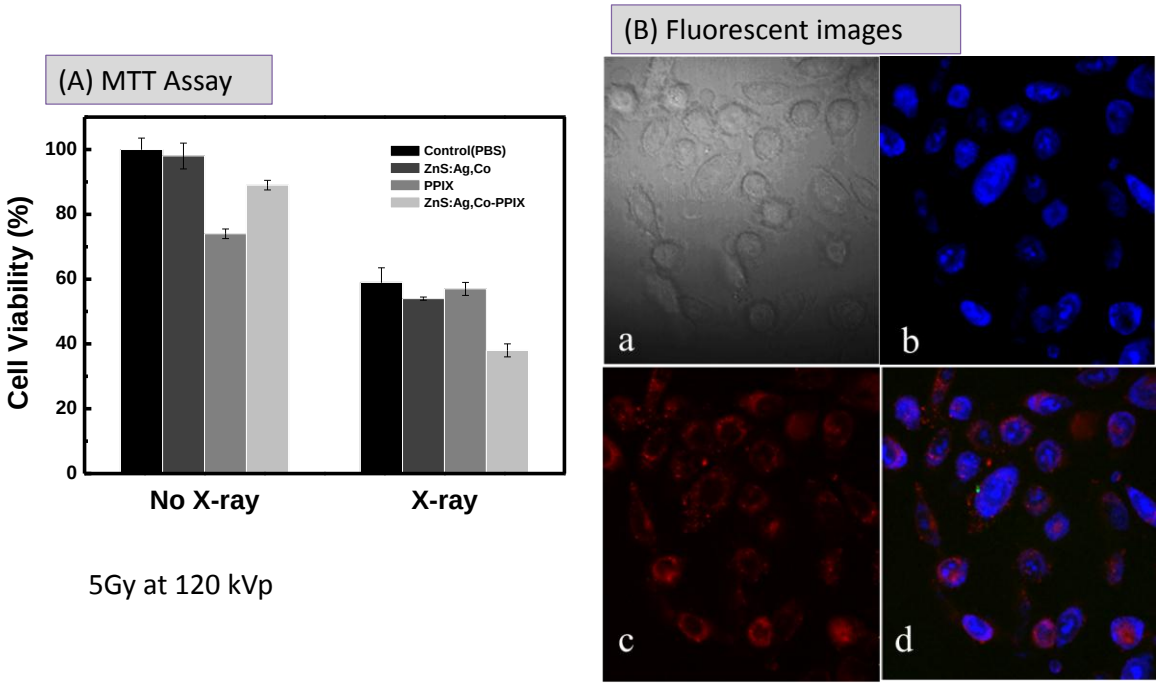


Figure 10. *In vitro* experiment of afterglow nanoparticle tagged with PPIX for cancer treatement. (A). MTT assay. With x-ray irradiation, PPIX tagged afterglow nanoparticles show maximum cell killing effect compared to other

groups. (B). Fluorescence images. (a) bright field image, (b) cell nuclei stained with blue, (c) distribution of afterglow nanoparticles, and (d) overlaid image of (c) and (d).

Reportable outcomes

The dose enhancement from gold nanoparticles has been evaluated using immunofluorescence, MTT, and clonogenic assays on prostate cancer cell lines (DU-145, PC-3, and LNCap), breast cancer cell line (MDA-MB-231), and cervix cancer cell line (HeLa). Different from our hypothesis, prostate cancer cell lines did not present dose enhancement from 50 nm gold nanoparticles with 105 kVp and 225 kVp x-ray irradiation in spite of many experiments of various protocols and settings. In addition, PC-3 and LNCap cell lines were not ideal cell lines for clonogenic assay because of their inability for colony formation in *in vitro* environment. With 100 nm gold nanopariticles at the same concentration as 50 nm gold nanoparticles being used, DU-145 could not form colonies at all. Concentration may need to be reduced considering the size of 100 nm gold nanoparticles. More study is necessary to optimize the concentration for DU-145 prostate cancer cell line and others. Unlike the previous experiment with prostate cancer cell lines, breast cancer cell line (MDA-MB-231) presented the dose enhancement from 100 nm gold nanoparticles. However, from 50 nm gold nanoparticles, this cell line did not present the dose enhancement. The breast cancer cell line and 100 nm gold nanoparticles were employed to resolve the difficulties observed on the prostate cancer cell line experiments. Even though the dose enhancement was observed in breast cancer cell line (MDA-MB-231), we have obtained valuable experience and knowledge to continue to study the dose enhancement radiation therapy for prostate cancer cell. First, dose enhancement using gold nanoparticles is dependent on cell line. Since DU-145 cell line did not form colonies at all with 100 nm gold nanoparticles, optimization for gold nanoparticle concentratition needs to be performed to finalize this statement. Second, the dose enhancement is the function of nanoparticle size. With 50 nm nanoparticles, the dose enhancement was not observed in any cell lines that were selected in the study. Even though 50 nm is claimed to be the optimal size for this kind of study because of maximal cellular uptake, it should be discreetly claimed as our studies gave different results. Third, dose enhancement is not only contributed by intracellular nanoparticles, but also by extracellular nanoparticles. Most of literatures stated that dose enhancement is caused by Auger electron from internalized nanoparticles. However, our studies present that dose enhancement was occurred by extracellular nanoparticles. Therefore, this also needs to be confirmed by experiment as well as some simulations, such as monte carlo simulation.

Conclusion

We have investigated dose enhancement effect using gold nanoparticles which can be potentially employed in radiation treatment. In this project, prostate cancer cell lines were investigated in depth to demonstrate dose enhancement using gold nanoparticles with kilovoltage x-ray irradiater. Various assays following different protocols and parameters were employed to conduct this project. In contrast to our hypothesis, prostate cancer

cell lines that we selected have not shown dose enhancement. For the purpose of problem-solving, 100 nm gold nanoparticles and MDA-MB-231 breast cancer cell line were employed. From this combination, we have concluded that dose enhancement is the function of nanoparticle size, cell line, and nanoparticle location (in the intra-/extra-cellular space). Even though dose enhancement was observed in the different cell line from what we proposed, our experience lays the foundation on our future studies and will greatly contribute to optimization of the experimental setup for dose enhancement study on prostate cancer cell lines. The afterglow nanoparticles have capability to emit visible light to excite photosensitizer tagged on the particle surface. This type of nanoparticles has a potential to treat deeply seated tumors. As a medical physicist, I am currently doing Monte Carlo simulation to find fundamental source of dose enhancement from gold nanoparticles and have a plan to continue to do *in-vitro* and by extension, *in-vivo* experiment.

References

1. G. Frens, Controlled nucleation for the regulation of the particle size in monodisperse gold suspension, Nature (Lond.) 241, 20-22
2. D. Chithrani, and W. Chan, "Elucidating the Mechanism of Cellular Uptake and Removal of Protein-Coated Gold Nanoparticles of Different Sizes and Shapes," Nano Lett. 7, 1542-1550 (2007)
3. W. Haiss, N. Thanh, J. Aveyard, and D Fernig, "Determination of Size Concentration of Gold Nanoparticles from UV-Vis Spectra", Anal. Chem. 79, 4215-4221 (2007)
4. D. Chithrani, S. Jelveh, F. Jalali, M. Prooijen, C. Allen, R. Bristow, R. Hill, and D. Jaffray, "Gold nanoparticles as radiation Sensitizers in Cancer Therapy", Radiation Research, 173, 719-728 (2010)
5. E. Cho, J. Xie, P. Wurm, and Y. Xia, "Understanding the Role of Surface Charges in Cellular Adsorption versus internalization by Selectively Removing Gold Nanoparticles on the Cell surface with a I2/KI Etchant," Nano Lett. 9, 1080-1084, (2009)